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# Induction of streptomycin resistance in the wild tomato *Lycopersicon peruvianum*

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**Summary.** A protoplast mutagenesis and cell selection system was used for the isolation of streptomycin resistant *Lycopersicon peruvianum* colonies. Protoplasts were treated with the mutagen N-nitroso-methylurea and could be regenerated into fertile plants, carrying the streptomycin resistant character. Several classes of streptomycin resistance could be distinguished. Reciprocal crosses between streptomycin resistant and sensitive plants showed a non-Mendelian transmission of the resistance trait. Streptomycin resistance is the first selectable and maternally inherited cell organelle marker described in tomato.

**Key words:** *Lycopersicon peruvianum* – Streptomycin resistance – Mutagen NMU – Protoplasts – Chloroplast marker

## Introduction

In most higher plants, including tomato, organelles are inherited uniparentally. Thus, combining cytoplasmic organelles with different genetic traits is not possible by sexual hybridization. Somatic cybridization in higher plants, however, provides opportunities to transfer cytoplasmic organelles between plant species and to investigate new nuclear-organelle and organelle-organelle interactions. An advantage for the analysis of organelle transfer and interaction in cybrids is the presence of selectable and easily screened genetic markers on organelles.

The tomato genome has been well characterized (Rick and Yoder 1988; Hille et al. 1989). In comparison with ancestral taxa the genetic variability of the cultivated tomato is limited. Several valuable genes encoding disease and pest resistances, e.g. the tobacco mosaic virus resistance gene, Tm-2a, and the root-knot nematode resistance Mi-gene, have been bred from wild *Lycopersicon* species in the cultivated tomato *L. esculentum* (Rick et al. 1987). On the contrary cytoplasmic genetic information from wild relatives could not be transmitted, owing to unilateral incongruity between the species. Therefore, transfer of cytoplasmic organelles from wild relatives to the cultivated tomato was attempted by somatic hybridization (O'Connell and Hanson 1986, 1987). A lack of selectable cell organelle markers in tomato species restricted the efficiency of selecting for organelle transfer.

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Several antibiotic resistance markers, such as lincomycin, spectinomycin and streptomycin resistance were used in genetic studies with *Chlamydomonas* and were localized in the chloroplast genome (Harris et al. 1977; Barlett et al. 1979; Lemieux et al. 1984). In higher plants, mutants resistant to lincomycin (Cseplo and Maliga 1982, 1984) and spectinomycin (Fluhr et al. 1985) were reported in tobacco species. These mutants possessed a single base-pair change in the 23S rRNA gene (lincomycin resistance; Cseplo et al. 1988) or 16S rRNA gene (spectinomycin resistance; Fromm et al. 1987) of the chloroplast genome. Streptomycin resistance is the most extensively used marker in higher plants and has already been induced in *Onobrychis viciifolia* (Hamill et al. 1986), *Petunia hybrida* (Binding et al. 1970) and *Nicotiana* species (Maliga et al. 1973; Fluhr et al. 1985; Umiel and Goldner 1976). Streptomycin binds to the 30S ribosomal subunit of procaryotic-like ribosomes, inhibits polypeptide synthesis and causes misreading of the genetic code (Davis et al. 1974; Gorini and Davies 1968; Edwards 1980). Single base-pair changes leading to streptomycin resistance were mapped to positions in the rps12 and/or 16S rRNA genes of the chloroplast genome in *Chlamydomonas reinhardtii* (Lemieux et al. 1984; Lemieux and Lee 1987; Gauthier et al. 1988), *Euglena gracilis* (Montandon 1985) and *Nicotiana* taxa (Fromm et al. 1987, 1989; Etzold et al. 1987). In *Escherichia coli,* streptomycin resistance was linked to comparable positions (Ozaki et al. 1969; Funatsu and Wittmann 1972; Montandon et al. 1986; Melancon et al. 1988). On the other hand streptomycin resistance can be controlled by a recessive nuclear mutation, as in *Nicotiana* (Maliga 1981) and *Chlamydomonas* (Harris et al. 1977; Lee and Jones 1973).

In tobacco streptomycin, spectinomycin, and lincomycin resistance and other chloroplast encoded mutations could be induced with the use of the mutagens nitroso-methylurea (NMU) or nitroso-ethylurea (NEU) (Fluhr et al. 1985; Cseplo et al. 1982, 1984; Maliga et al. 1981; Hagemann 1982; Hosticka and Hanson 1984). If by analogy to other species, a C–T transition is required to obtain streptomycin resistance in tomato, NMU is a potential inducer of this transition (Richardson et al. 1987). In tomato species, selectable cytoplasmic markers have not yet been reported. In this paper we describe the induction of streptomycin resistance in the wild tomato species *L. peruvianum.* The experiments were performed with and without the use of NMU.

## Materials and methods

*Materials.* Streptomycin sulfate (Sigma) was filter sterilized before addition to media. *L. peruvianum* PI 126929 was obtained from the Institute for Horticultural Plant Breeding (IVT), Wageningen. Plants were propagated in sterile glass containers on MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 0.9% Difco agar and grown at 25° C, 1500 lux, 16/8 h photoperiod and 60% humidity.

*Chemical mutagenesis.* A solution of 20 mM NMU (Sigma) was prepared just before use in citric-phosphate buffer (Hagemann 1982). The solution was filter sterilized, diluted in protoplast culture medium and added to freshly isolated protoplasts at a final concentration of 0–1 mM. NMU is subject to hydrolytic degradation in aqueous solutions (Hagemann 1982) and, therefore, does not need to be washed out.

*Protoplast isolation.* Leaf mesophyll protoplasts were isolated from in vitro grown *L. peruvianum* plants. Leaves were cut into small sections and incubated in a preplasmolysing solution [CPW salts (Frearson et al. 1973), 7.5% mannitol, 3 mM MES, pH 5.8] for 1 h in the dark. The solution was replaced by the same solution supplemented with 1% cellulase R10 (Yakult) and 0.15% macerozyme (Yakult) and incubated for 18 h in the dark at 25° C. After enzyme incubation the mixture was diluted with an equal volume of washing medium (CPW salts, 2% KCl) and filtered through a 70 µM nylon sieve. The filtrate was centrifuged for 5 min at 800 rpm and the pellet was resuspended in 6 ml washing medium. The protoplasts were purified by flotation on a CPW 18% sucrose solution after centrifugation for 5 min at 1000 rpm. The protoplasts were then washed twice by centrifugation in washing medium for 5 min at 800 rpm and counted in a haemocytometer.

*Protoplast culture.* Protoplasts were cultured in 1/2 VKM medium (Binding and Nehls 1977) at a density of $10^5$ protoplasts/ml in 9-cm petri dishes. The cultures were diluted twice weekly with protoplast medium containing 0.75 mg/l benzylaminopurine (BAP). After 3–4 weeks in liquid culture (dark, 25° C) the colonies were embedded in 0.8% agarose. The agar was cut into pieces and transferred to 15 ml of liquid medium consisting of B5 micro/macro salts (Gamborg et al. 1968), Nitsch vitamins (Nitsch 1961), 0.2 M mannitol, 7.3 mM sucrose, 0.5 mg/l BAP, 0.05 mg/l naphthylacetic acid (NAA) and supplemented with 500 mg/l streptomycin.

*Plant regeneration.* Green colonies that developed on 500 mg/l streptomycin were isolated and subcultured on the same medium. Plants were regenerated from the selected colonies by transferring them to subsequently shoot-inducing medium [MS salts, Nitsch vitamins, 0.2 M mannitol, 7.3 mM sucrose, 2 mg/l zeatin, 0.2 mg/l indoleacetic acid (IAA)] and shoot regeneration medium (MS salts, 2% sucrose, 2 mg/l zeatin, 0.2 mg/l IAA) both containing 500 mg/l streptomycin. Shoots were rooted on MS medium with 2% sucrose and 250 mg/l streptomycin. Rooted plants were transferred to soil and grown to maturity in the greenhouse.

*Protoplast plating efficiency.* Plating efficiencies were estimated by counting dividing colonies after 10 days of culture in 1/32 of the circle from a petri dish and dividing this value by the number of protoplasts originally added to the petri dish.

*Leaf assay.* Leaf sections were cut from in vitro grown plants and incubated for shoot induction on MS media containing 2% sucrose, 2 mg/l zeatin, 0.2 mg/l IAA and 0–1000 mg/l streptomycin. Streptomycin resistance was determined by the ability of explants to form shoots on streptomycin containing media.

*Seedling assay.* Seeds were surface sterilized with a 1% hypochlorite solution for 10 min, followed by several washing steps with sterile water. The seeds were germinated in sterile culture on MS medium containing 2% sucrose and 250 mg/l streptomycin.

*Ploidy level.* Leaf epidermal strips from in vivo or in vitro plants were treated with $KI/I_2$. The number of chloroplasts per guard cell pair was counted using a bright field microscope. The average number of chloroplasts in ten guard cell pairs was taken as a measure of ploidy level (Koornneef et al. 1989).

## Results

### *Effect of streptomycin*

The sensitivity of *L. peruvianum* to the antibiotic streptomycin was determined at several levels of plant cell development. Streptomycin severely restricted the rooting and

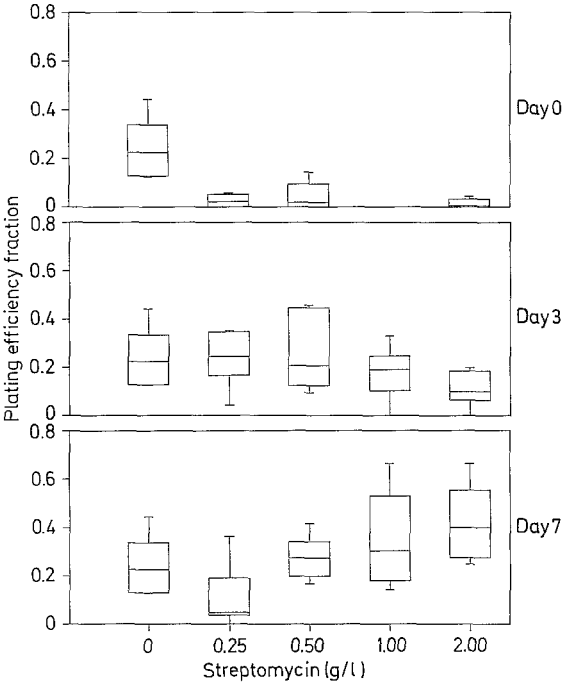


**Fig. 1.** The effect of streptomycin on the plating efficiency of protoplasts. Streptomycin was added at several concentrations to protoplast cultures at 0, 3 and 7 days after isolation. Box-and-whisker plots (Tukey 1977) give the fraction of dividing protoplasts per incubated protoplasts for each streptomycin concentration. The *box* shows the location of the hinges or quartiles. The end of the whiskers denote the outermost values, unless there are outside values, which are marked with a *star* (see Fig. 2)

growth of seedlings. Leaf explants grown on regeneration medium containing streptomycin at different concentrations did neither develop callus growth nor formed shoots on media with increasing streptomycin concentrations. Streptomycin inhibited greening of protoplast derived calli at concentrations from 250 mg/l. Concentrations of 1000 mg/l streptomycin also affected the growth of these calli. The effect of streptomycin on protoplasts was dependent on the moment of selection: no division of protoplasts was observed when streptomycin was added to freshly isolated protoplasts (Fig. 1). No effect on the plating efficiency, but only a retardation of callus growth could be detected when streptomycin was added 7 days after protoplast isolation.

*Selection of streptomycin resistant colonies*

*L. peruvianum* protoplasts ($28 \times 10^6$) were treated with several concentrations of NMU directly after isolation. An estimation was made of the genotoxic effects of the mutagen by measuring the capacity of the protoplasts to divide after application of NMU. A decline in the plating efficiency of the protoplasts was detected with increasing concentrations of NMU, as shown in the dose-response curve (Fig. 2). This effect was highly significant. Following mutagenesis the selection of streptomycin resistant colonies started 4 weeks later. Streptomycin resistant colonies were isolated by their ability to green on selective medium containing 500 mg/l streptomycin. Green calli could be observed following 2 months of culture in selective medium. Resistant calli were retested and then subcultured on regeneration

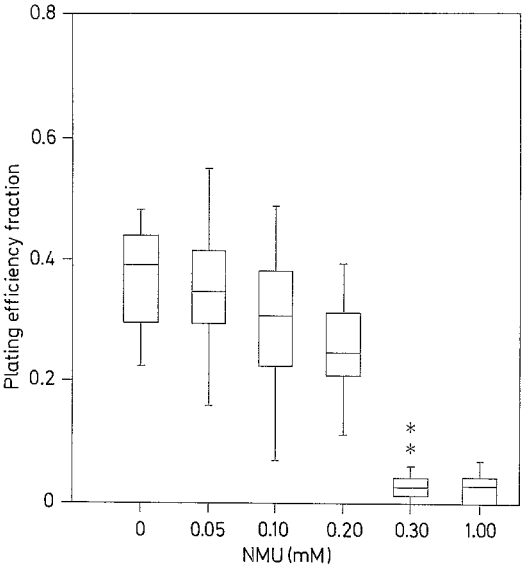


**Fig. 2.** Dose-response curve of nitroso-methylurea (NMU) and *Lycopersicon peruvianum* protoplasts. NMU was added at several concentrations to freshly isolated protoplasts. The plating efficiency was determined as described in the Materials and methods. See the legend to Fig. 1 for explanation of the box-and-whisker plot

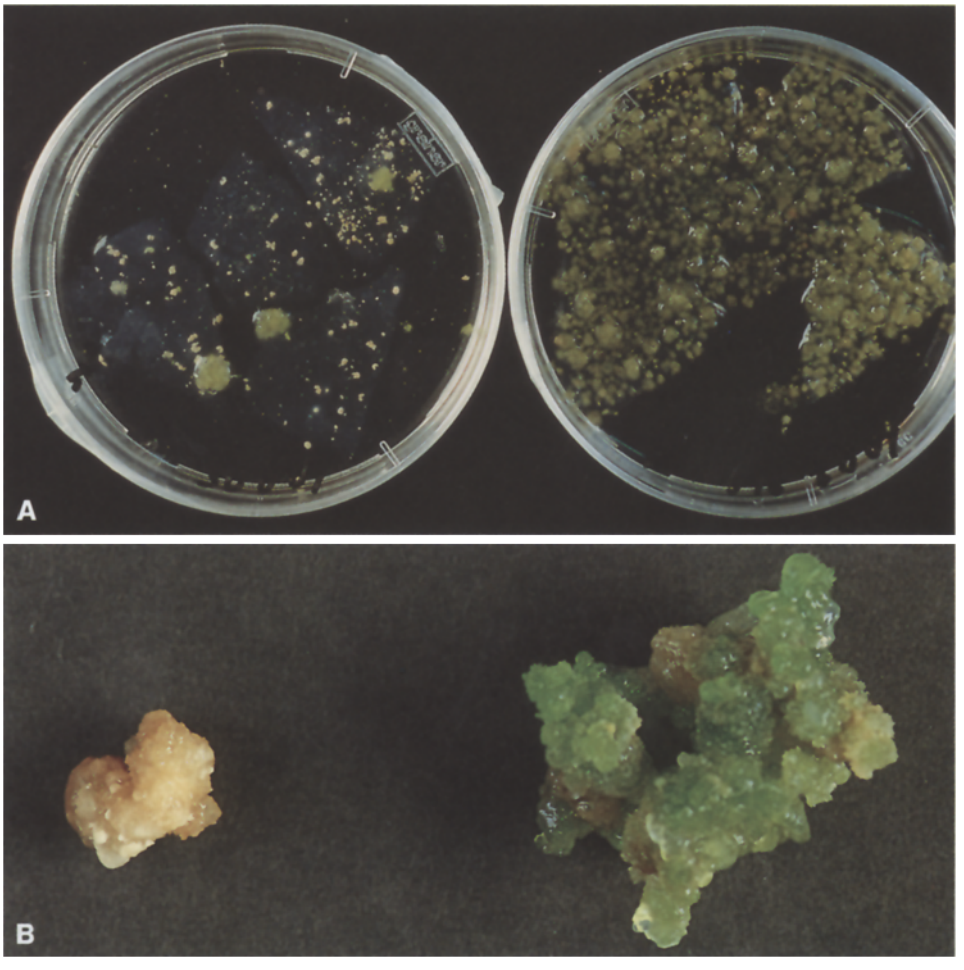


**Fig. 3A and B.** The development of streptomycin resistant colonies. Protoplasts were plated on control (right) and selective medium (left) containing 500 mg/l streptomycin at day 28. **A** After 2 months, streptomycin resistant calli appeared on the selective medium. **B** Several subcultures later stable green streptomycin resistant calli could be isolated (*right*), in contrast to control streptomycin sensitive calli (*left*) which did not develop on the selective medium (500 mg/l streptomycin)

**Table 1.** Frequency of streptomycin resistant (strR) colonies after addition of nitroso-methylurea (NMU) in several concentrations to freshly isolated protoplasts[a]

| NMU (mM) | No. of protoplasts ($\times 10^6$) | No. of colonies ($\times 10^6$) | No. of strR colonies | No. of strR colonies as fraction of | |
|---|---|---|---|---|---|
| | | | | Protoplasts ($\times 10^{-6}$) | Colonies ($\times 10^{-6}$) |
| 0 | 0.4 | 0.05 | 0 | 0 | 0 |
| 0.05 | 4.6 | 0.58 | 7 | 1.5 | 12 |
| 0.1 | 4.0 | 0.22 | 53 | 13.3 | 241 |
| 0.2 | 4.6 | 0.18 | 26 | 5.7 | 144 |
| 0.3 | 4.2 | 0.11 | 21 | 5.0 | 191 |
| 1.0 | 5.2 | 0 | 0 | 0 | – |

[a] Frequency was determined after 1 year of selection on streptomycin

medium in the presence of streptomycin. The mode of selection of streptomycin resistant clones is shown in Fig. 3.

The occurrence and distribution of streptomycin resistant calli was determined for each NMU concentration used. The mutation frequency was calculated as the number of both mutagenized protoplasts and surviving mutagenized protoplasts (Table 1). NMU increased the number of mutants, with rising concentrations of mutagen. An optimal mutation frequency of $1.3 \times 10^{-5}$, calculated on the basis of the number of mutagenized protoplasts, was obtained when 0.1 mM NMU was used. In other experiments an attempt was made to obtain streptomycin resistant clones without the use of NMU. In this case $20 \times 10^6$ protoplasts were cultured in the presence of streptomycin. Just one streptomycin resistant clone could be isolated by somaclonal variation and this implies that a mutation frequency of $0.5 \times 10^{-7}$, calculated on the basis of the number mutagenized protoplasts, was obtained.

*Regeneration of streptomycin resistant calli*

The first shoots were obtained 6 months after protoplast isolation and cultured in the presence of streptomycin. Plant regeneration was obtained in 113 out of 123 streptomycin resistant calli. No effect of NMU on the regeneration capacity was detected, since approximately all calli regenerated shoots.

*Properties of the streptomycin resistant clones*

An assay was done on the occurrence of streptomycin dependent colonies. Streptomycin resistant calli were inoculated on media with and without the addition of streptomycin. Growth of streptomycin dependent colonies was only expected in the presence of streptomycin. No streptomycin dependent clones were observed among 105 calli tested.

The regenerates of the streptomycin resistant calli were tested for rooting capability in the presence and absence of streptomycin as an extra control on resistance. Several classes of root growth could be distinguished (Fig. 4). The levels of streptomycin resistance varied from total resistance (normal root growth) to that only slightly less sensitive than the sensitive wild-type (growth, but no rooting), whereas in the absence of streptomycin rooting of all shoots was normal.

The stability of streptomycin resistance was studied by a leaf assay. The plants regenerated from the resistant callus retained streptomycin resistance, as indicated by the appearance of shoots on leaf sections of the regenerates on selective medium containing concentrations of up to 1 mg/ml streptomycin (Fig. 5). The streptomycin resistant plants were normal with respect to morphology. The ploidy level of streptomycin resistant regenerates was determined: among a total of 25 plants, 22 were diploid. Cross-resistance to the antibiotics kanamycin, lincomycin and hygromycin was also determined using the leaf assay: no cross-resistance was found to these antibiotics.


**Fig. 4. A** Several classes of root growth of streptomycin resistant colonies. Shoot cuttings were incubated on MS 20 media containing 250 mg/l streptomycin. Four classes of root growth of streptomycin resistant regenerates are shown. **B** The distribution of streptomycin resistant regenerates among the several classes of root growth obtained. − = Growth, no roots; + = many short roots; ++ = a small quantity of normal roots; +++ = normal roots

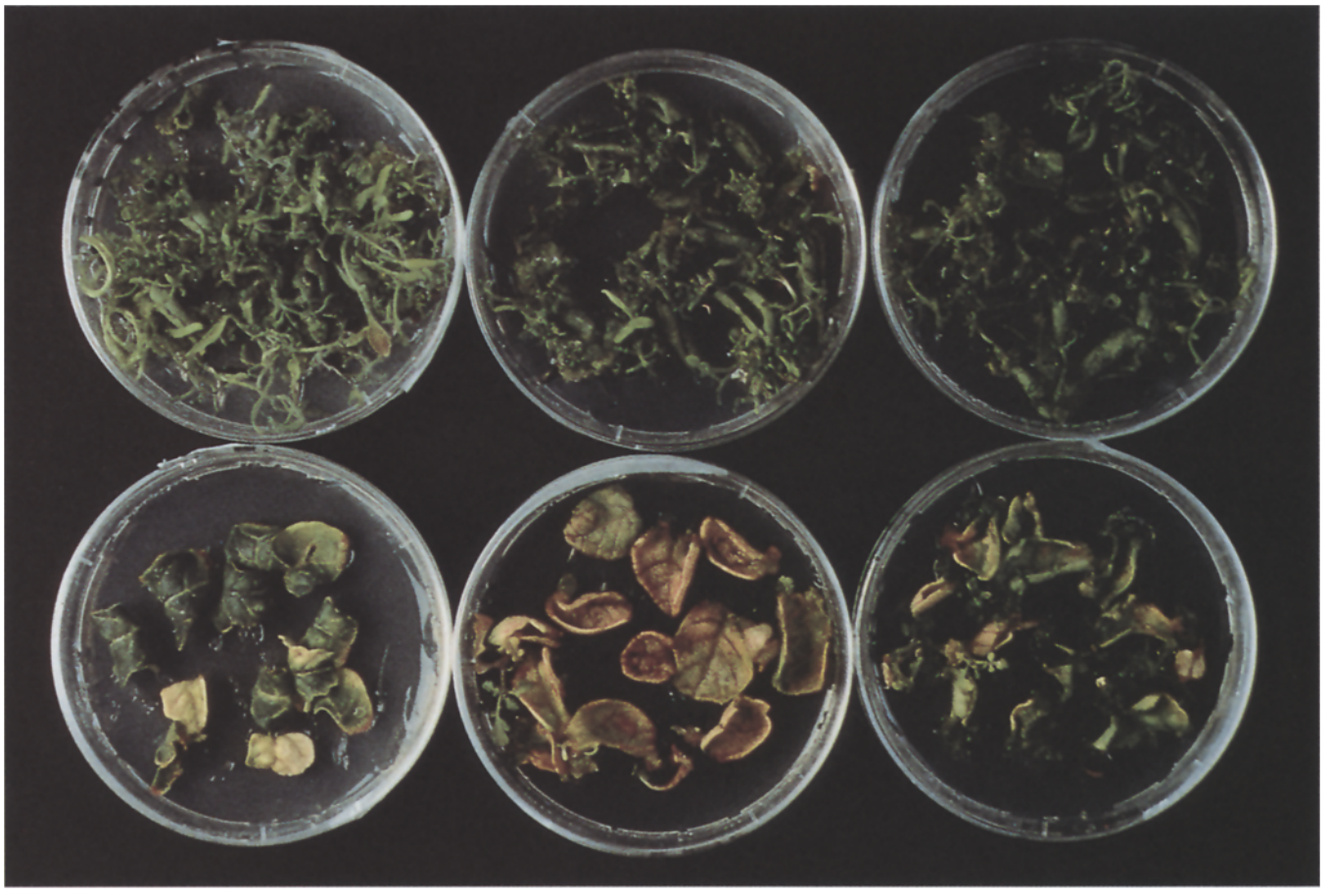

**Fig. 5.** Streptomycin resistance test with leaf sections of a regenerated streptomycin resistant plant (*above*) and a streptomycin sensitive control (*below*). Leaf explants were put on regeneration medium, containing from right to left resp. 0–250–500 mg/l streptomycin

*Inheritance of streptomycin resistance*

The transmission of streptomycin resistance to the progeny was studied as evidence for the mutational origin of streptomycin resistance. Reciprocal crosses were made between one flowering streptomycin resistant plant and the original streptomycin sensitive plants. Plants were scored as streptomycin resistant when seedlings showed normal growth and rooting on media containing 250 mg/l streptomycin. When the streptomycin resistant plant was the female parent, all of the offspring were streptomycin resistant (Table 2). On the contrary all progeny were streptomycin sensitive when the pollen of the resistant plant was used. These results confirm the genetic nature of the streptomycin resistance trait and demonstrate that streptomycin resistance is controlled by a maternally inherited mutation.

**Table 2.** The inheritance of streptomycin resistance. Reciprocal crosses were performed with streptomycin resistant (R) and streptomycin sensitive (S) plants. Seeds from M2 plants were germinated on MS 20 media containing 250 mg/l streptomycin. Resistant and sensitive seedlings were discriminated on account of root growth

| Cross | % Germination | Number of seedlings tested | |
|---|---|---|---|
| | | Resistant | Sensitive |
| L. per (R) × L. per (S) | 81 | 720 | 0 |
| L. per (S) × L. per (R) | 91 | 0 | 380 |

## Discussion

We were able to isolate 123 streptomycin resistant colonies, making use of NMU. Via somaclonal variation only one streptomycin resistant clone could be isolated from practically the same amount of protoplasts. The frequency of resistant clones was in the order of $10^{-7}$ in the latter case, which corresponds to values reported for the spontaneous frequency of streptomycin resistance ranging from $1 \times 10^{-9}$ (Fluhr et al. 1985) to $1 \times 10^{-6}$ (Maliga et al. 1973; Hamill et al. 1986). Calculated in the same way, NMU increased the frequency of streptomycin resistant colonies up to $10^{-5}$. In our experiments NMU proved to be an efficient mutagen, compared to values reported for NEU. NEU was shown to increase 30-fold the appearance of streptomycin resistant clones derived from protoplasts of *Nicotiana plumbaginifolia* (Maliga et al. 1981) and in case of chlorate resistant clones in the same system this value was increased 25-fold (Marton et al. 1982). Most experiments on mutagenesis showed increasing frequency of variants with increasing mutagen dose (Sung 1976; Weber and Lark 1980). High doses of mutagen, however, induce abnormalities and affect the ability to regenerate plants. Using protoplast-derived colonies, Caboche and Muller (1980) have demonstrated that the number of colonies able to regenerate decreased proportionally with the dose of mutagen. In our experiments a NMU concentration of 0.1 mM was a good compromise. This supports the hypothesis that efficient mutagenic treatments do not require doses inducing high kill-

ing rates, and we also observed no effect of the NMU dose on regeneration capacity. No abnormal plantlets were isolated and no pigment deficient phenotypes were detected, as described by Cseplo and Maliga (1984).

An alteration of a chloroplast component might be encoded by either the chloroplast or nuclear genome. The inheritance pattern of streptomycin resistance was studied in one streptomycin resistant regenerate to determine the mutational origin. The results from reciprocal crosses confirmed that the resistance is due to a mutation rather than epigenetic phenomena and showed that streptomycin resistance was transmitted in a non-Mendelian, uniparental way, as expected. Both Mendelian and non-Mendelian inherited mutations of streptomycin resistance have been induced in *Chlamydomonas* (Gillham 1965; Lee and Jones 1973) and *Nicotiana* (Maliga et al. 1975; Maliga 1981; Umiel 1979), but nuclear-encoded streptomycin resistance always inherited as a recessive trait and was only isolated using haploid plant material. Thus, it was unlikely to expect a dominant nuclear streptomycin-resistant mutant and, even less likely, a homozygous recessive nuclear mutation in diploid plant material. Four classes of streptomycin resistance have been identified by the root morphology of regenerates on streptomycin containing medium. This phenomenon may be correlated with the site of the induced mutation, as streptomycin resistance can result from different mutations in the 16S rRNA or associated ribosomal protein genes. The existence of several phenotypes with regard to the responses to streptomycin concentrations in the medium were reported in *Nicotiana* (Umiel 1976; Maliga 1981; Fluhr et al. 1985), *E. coli* (Wittmann and Wittmann-Liebold 1974) and *Chlamydomonas* (Lee et al. 1973; Harris et al. 1977). In *Chlamydomonas* three levels of resistance against streptomycin were observed, which could be assigned to four different loci for streptomycin resistance in the chloroplast genome (Harris et al. 1977). A high level of resistance was reported in *E. coli,* in which case the mutation was located in the gene encoding the ribosomal protein S12. It can be surmised that a mutation in the S12 gene results in a higher level of resistance compared with mutations in other protein genes or the 16S rRNA gene. In this respect the positions of the mutations leading to streptomycin resistance mutations in our material await determination.

No cross-resistance of the streptomycin resistant clones to other antibiotics could be determined. Cross-resistance between streptomycin and lincomycin was also not observed by Cseplo and Maliga (1982). Cross-resistance between kanamycin and streptomycin resistance was reported (Dix et al. 1977), but could not be confirmed in our experiments. In *Euglena* no cross-resistance was found between streptomycin, kanamycin and clindamycin (a lincomycin derivative) (Nicolas 1981). In the case of *Chlamydomonas* cross-resistance was detected between streptomycin and neamine (Gillham 1965).

The streptomycin resistant colonies were tested for streptomycin dependence. Conditionally streptomycin dependent (CSD) mutants were described in *E. coli, Salmonella* (Gorini and Davies 1968) and *Chlamydomonas* (Gillham 1965) but not in higher plants. An explanation for the absence of streptomycin dependent clones in tomato protoplast culture can be of two kinds. Firstly, diploid plant material was used and therefore no recessive auxotrophic mutants are expected. Secondly, the mode of selection of streptomycin resistance that we applied is important. When auxotrophic mutations did appear these mutants would have been lost in the minimal medium used to culture protoplasts during the first 4 weeks. One must be aware that streptomycin resistant mutations can disappear during the period when no selection pressure is performed. On the other hand it can be envisaged that time is needed for segregation of the mutational event before selection pressure can be withstood. The first resistant clones appeared after 2 months of selection and after a next selection cycle a stable population of streptomycin resistant colonies was obtained. The stability of streptomycin resistance was analysed in various stages of differentation and could be demonstrated in all cases.

During the preparation of this manuscript McCabe et al. (1989) reported the isolation of streptomycin resistant *L. peruvianum* plants; however, these were not genetically characterized. From a comparison between their method and our system the following conclusions can be made. With regard to the regeneration of induced mutants their method would allow fewer problems, as the regeneration of shoots from protoplast derived calli is still limited for most species. On the other hand we isolated several classes of mutants, and this implies that a wider spectrum of mutants might be obtained by using protoplasts for mutation induction. By using leaf explants as sources of mutation induction only those variants can be obtained that are expressed at that organizational level. Apart from this consideration the selection of mutants in our system is the same as the mode of selection applied after fusing protoplasts using the mutation as marker, which was the purpose of both studies.

In summary, efficient selection of mutants from protoplast cultures of *Lycopersicon peruvianum* can be obtained using NMU. This allows a systematic isolation of chloroplast-encoded antibiotic resistant mutants from protoplasts that are able to regenerate. In fact large-scale experiments have already been performed successfully for the isolation of lincomycin- and streptomycin-resistant mutants in other *Lycopersicon* species (Glas et al. in preparation; Jansen et al. in preparation). These mutants might prove to be of great value both in studies on the genetics of cytoplasmic organelles and in the development of selection systems for somatic hybrids.

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